

B/2

## (12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
16 January 2003 (16.01.2003)

PCT

(10) International Publication Number  
WO 03/004598 A2

(51) International Patent Classification: C12N

(21) International Application Number: PCT/EP02/06565

(22) International Filing Date: 14 June 2002 (14.06.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

101 32 946.6 6 July 2001 (06.07.2001) DE  
60/303,790 10 July 2001 (10.07.2001) USCZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,  
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG,  
SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ,  
VN, YU, ZA, ZM, ZW.(84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),  
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR,  
GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent  
(BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR,  
NE, SN, TD, TG).(71) Applicant (*for all designated States except US*): DE-  
GUSSA AG [DE/DE]; Bennigsenplatz 1, 40474 Düssel-  
dorf (DE).

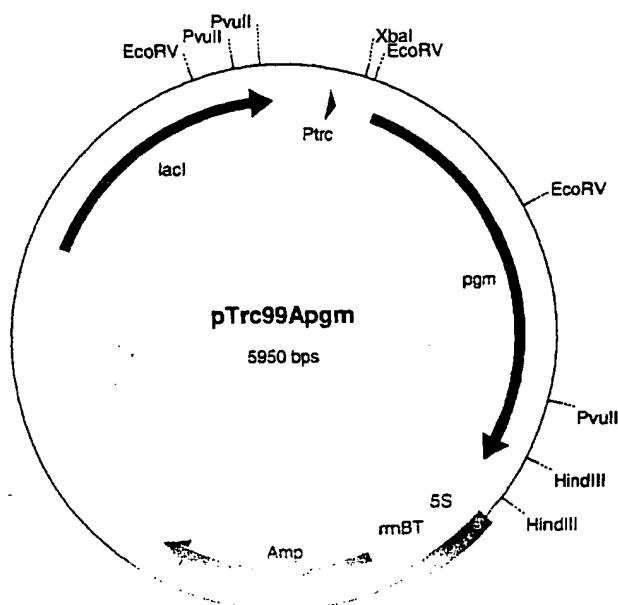
Declaration under Rule 4.17:

— of inventorship (Rule 4.17(iv)) for US only

(72) Inventor; and

(75) Inventor/Applicant (*for US only*): RIEPING, Mechthild  
[DE/DE]; Mönkebergstrasse 1, 33619 Bielefeld (DE).

Published:

— without international search report and to be republished  
upon receipt of that report(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE  
FAMILY

03/004598 A2

(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine.

**Process for the Preparation of L-Amino Acids using Strains  
of the Enterobacteriaceae Family**

Field of the Invention

This invention relates to a process for the preparation of  
5 L-amino acids, in particular L-threonine, using strains of  
the Enterobacteriaceae family in which at least the pgm  
gene is enhanced.

Prior Art

L-Amino acids, in particular L-threonine, are used in human  
10 medicine and in the pharmaceuticals industry, in the  
foodstuffs industry and very particularly in animal  
nutrition.

It is known to prepare L-amino acids by fermentation of  
strains of Enterobacteriaceae, in particular Escherichia  
15 coli (E. coli) and Serratia marcescens. Because of their  
great importance, work is constantly being undertaken to  
improve the preparation processes. Improvements to the  
process can relate to fermentation measures, such as e.g.  
stirring and supply of oxygen, or the composition of the  
20 nutrient media, such as e.g. the sugar concentration during  
the fermentation, or the working up to the product form, by  
e.g. ion exchange chromatography, or the intrinsic output  
properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are  
25 used to improve the output properties of these  
microorganisms. Strains which are resistant to  
antimetabolites, such as e.g. the threonine analogue  $\alpha$ -  
amino- $\beta$ -hydroxyvaleric acid (AHV), or are auxotrophic for  
metabolites of regulatory importance and produce L-amino  
30 acid, such as e.g. L-threonine, are obtained in this  
manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of strains of the Enterobacteriaceae family which produce L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating the effect on the production.

#### Object of the Invention

The object of the invention is to provide new measures for improved fermentative preparation of L-amino acids, in particular L-threonine.

#### Summary of the Invention

The invention provides a process for the preparation of L-amino acids, in particular L-threonine, using microorganisms of the Enterobacteriaceae family which in particular already produce L-amino acids and in which the nucleotide sequence which codes for the pgm gene is enhanced.

#### Detailed Description of the Invention

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Threonine is particularly preferred.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or a gene or allele which codes for a corresponding enzyme or

protein with a high activity, and optionally combining these measures.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is  
5 in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

10 The process is characterized in that the following steps are carried out:

- a) fermentation of microorganisms of the Enterobacteriaceae family in which the *pgm* gene is enhanced,
- 15 b) concentration of the corresponding L-amino acid in the medium or in the cells of the microorganisms of the Enterobacteriaceae family, and
- c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its  
20 entirety or portions (> 0 to 100%) thereof optionally remaining in the product.

The microorganisms which the present invention provides can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally  
25 cellulose or from glycerol and ethanol. They are representatives of the Enterobacteriaceae family chosen from the genera *Escherichia*, *Erwinia*, *Providencia* and *Serratia*. The genera *Escherichia* and *Serratia* are preferred. Of the genus *Escherichia* the species *Escherichia*  
30 *coli* and of the genus *Serratia* the species *Serratia marcescens* are to be mentioned in particular.

Suitable strains, which produce L-threonine in particular, of the genus *Escherichia*, in particular of the species *Escherichia coli*, are, for example

- Escherichia coli TF427
- 5 Escherichia coli H4578
- Escherichia coli KY10935
- Escherichia coli VNIIGenetika MG442
- Escherichia coli VNIIGenetika M1
- Escherichia coli VNIIGenetika 472T23
- 10 Escherichia coli BKIIM B-3996
- Escherichia coli kat 13
- Escherichia coli KCCM-10132.

Suitable L-threonine-producing strains of the genus *Serratia*, in particular of the species *Serratia marcescens*,  
15 are, for example

- Serratia marcescens* HNr21
- Serratia marcescens* TLr156
- Serratia marcescens* T2000.

Strains from the Enterobacteriaceae family which produce L-  
20 threonine preferably have, inter alia, one or more genetic or phenotypic features chosen from the group consisting of: resistance to  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to  $\alpha$ -methylserine, resistance to diaminosuccinic acid,  
25 resistance to  $\alpha$ -aminobutyric acid, resistance to borrelidin, resistance to rifampicin, resistance to valine analogues, such as, for example, valine hydroxamate, resistance to purine analogues, such as, for example, 6-dimethylaminopurine, a need for L-methionine, optionally a  
30 partial and compensable need for L-isoleucine, a need for meso-diaminopimelic acid, auxotrophy in respect of threonine-containing dipeptides, resistance to L-threonine, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid,

resistance to L-aspartate, resistance to L-leucine,  
resistance to L-phenylalanine, resistance to L-serine,  
resistance to L-cysteine, resistance to L-valine,  
sensitivity to fluoropyruvate, defective threonine  
5 dehydrogenase, optionally an ability for sucrose  
utilization, enhancement of the threonine operon,  
enhancement of homoserine dehydrogenase I-aspartate kinase  
I, preferably of the feed back resistant form, enhancement  
of homoserine kinase, enhancement of threonine synthase,  
10 enhancement of aspartate kinase, optionally of the feed  
back resistant form, enhancement of aspartate semialdehyde  
dehydrogenase, enhancement of phosphoenol pyruvate  
carboxylase, optionally of the feed back resistant form,  
enhancement of phosphoenol pyruvate synthase, enhancement  
15 of transhydrogenase, enhancement of the RhtB gene product,  
enhancement of the RhtC gene product, enhancement of the  
YfiK gene product, enhancement of a pyruvate carboxylase,  
and attenuation of acetic acid formation.

It has been found that microorganisms of the  
20 Enterobacteriaceae family produce L-amino acids, in  
particular L-threonine, in an improved manner after  
enhancement, in particular over-expression, of the pgm  
gene.

The use of endogenous genes is in general preferred.  
25 "Endogenous genes" or "endogenous nucleotide sequences" are  
understood as meaning the genes or nucleotide sequences  
present in the population of a species.

The nucleotide sequences of the genes of Escherichia coli  
belong to the prior art and can also be found in the genome  
30 sequence of Escherichia coli published by Blattner et al.  
(Science 277: 1453-1462 (1997)).

The following information on the pgm gene is known, inter  
alia, from the prior art:

Description: Phosphoglucomutase  
EC No.: 5.4.2.2  
Reference: Lu and Kleckner, Journal of Bacteriology  
176(18): 5847-5851 (1994) Brautaset et al.;  
5 Biotechnology and Bioengineering 58(2-3):  
299-302 (1998)  
Accession No.: AE000172

The nucleic acid sequences can be found in the databanks of the National Center for Biotechnology Information (NCBI) of  
10 the National Library of Medicine (Bethesda, MD, USA), the nucleotide sequence databank of the European Molecular  
Biologies Laboratories (EMBL, Heidelberg, Germany or Cambridge, UK) or the DNA databank of Japan (DDBJ, Mishima, Japan).

15 Alleles of the pgm gene which result from the degeneracy of the genetic code or due to "sense mutations" of neutral function can furthermore be used.

To achieve an enhancement, for example, expression of the genes or the catalytic properties of the proteins can be  
20 increased. The two measures can optionally be combined.

To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes  
25 which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative L-threonine production. The expression is likewise improved by measures to prolong the  
30 life of the m-RNA. Furthermore, the enzyme activity is also enhanced by preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome.

Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert,  
5 inter alia, in Chang and Cohen (Journal of Bacteriology 134: 1141-1156 (1978)), in Hartley and Gregori (Gene 13: 347-353 (1981)), in Amann and Brosius (Gene 40: 183-190 (1985)), in de Broer et al. (Proceedings of the National Academy of Sciences of the United States of America 80:  
10 21-25 (1983)), in LaVallie et al. (BIO/TECHNOLOGY 11: 187-193 (1993)), in PCT/US97/13359, in Llosa et al. (Plasmid 26: 222-224 (1991)), in Quandt and Klipp (Gene 80: 161-169 (1989)), in Hamilton (Journal of Bacteriology 171: 4617-4622 (1989)), in Jensen and Hammer (Biotechnology and  
15 Bioengineering 58: 191-195 (1998)) and in known textbooks of genetics and molecular biology.

Plasmid vectors which are capable of replication in Enterobacteriaceae, such as e.g. cloning vectors derived from pACYC184 (Bartolomé et al.; Gene 102: 75-78 (1991)),  
20 pTrc99A (Amann et al.; Gene 69: 301-315 (1988)) or pSC101 derivatives (Vocke and Bastia, Proceedings of the National Academy of Sciences USA 80(21): 6557-6561 (1983)) can be used. A strain transformed with a plasmid vector, wherein the plasmid vector carries at least one nucleotide sequence  
25 which codes for the pgm gene, can be employed in a process according to the invention.

It is also possible to transfer mutations which affect the expression of the particular gene into various strains by sequence exchange (Hamilton et al.; Journal of Bacteriology  
30 171: 4617 - 4622 (1989)), conjugation or transduction.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, with strains of the Enterobacteriaceae family to enhance one or more enzymes of the known threonine biosynthesis pathway or enzymes of

anaplerotic metabolism or enzymes for the production of reduced nicotinamide adenine dinucleotide phosphate, in addition to the enhancement of the *pgm* gene.

Thus, for example, one or more of the genes chosen from the  
5 group consisting of

- the *thrABC* operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),
- 10 • the *pyc* gene which codes for pyruvate carboxylase (DE-A-19 831 609),
- the *pps* gene which codes for phosphoenol pyruvate synthase (Molecular and General Genetics 231(2): 332-336 (1992)),
- 15 • the *ppc* gene which codes for phosphoenol pyruvate carboxylase (Gene 31: 279-283 (1984)),
- the *pntA* and *pntB* genes which code for transhydrogenase (European Journal of Biochemistry 158: 647-653 (1986)),
- the *rhtB* gene which imparts homoserine resistance (EP-A-0 994 190),
- 20 • the *mgo* gene which codes for malate:quinone oxidoreductase (WO 02/06459),
- the *rhtC* gene which imparts threonine resistance (EP-A-1 013 765),
- 25 • the *thrE* gene of *Corynebacterium glutamicum* which codes for the threonine export protein (WO 01/92545),
- the *gdhA* gene which codes for glutamate dehydrogenase (Nucleic Acids Research 11: 5257-5266 (1983); Gene 23: 199-209 (1983)),

- the dps gene which codes for the global regulator Dps (Genes & Development 6(12B): 2646-2654 (1992), Accession No. AE000183),
- 5 • the lrp gene which codes for the regulator of the leucine Lrp regulon and high-affinity transport systems of branched-chain amino acids (Journal of Biological Chemistry 266(17): 10768-10774 (1991), Accession No. AE000191),
- 10 • the hns gene which codes for the DNA-binding protein HLP-II (Molecular and General Genetics 212(2): 199-202 (1988), Accession No. AE000222),
- the fba gene which codes for fructose bisphosphate aldolase (Biochemical Journal 257: 529-534 (1989), Accession No. AE000376),
- 15 • the ptsG gene which codes for the glucose-specific IIBC component of the phosphotransferase system PTS (Journal of Biological Chemistry 261(35): 16398-16403 (1986), Accession No. AE000210),
- 20 • the ptsH gene of the ptsHIcrr operon which codes for the phosphohistidine protein hexose phosphotransferase of the phosphotransferase system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),
- 25 • the ptsI gene of the ptsHIcrr operon which codes for enzyme I of the phosphotransferase system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),
- 30 • the crr gene of the ptsHIcrr operon which codes for the glucose-specific IIA component of the phosphotransferase system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),

- the mopB gene which codes for chaperone GroES (Journal of Biological Chemistry 261(26): 12414-12419 (1986), Accession No. AE000487),
- 5 • the ahpC gene of the ahpCF operon which codes for the small subunit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences USA 92(17): 7617-7621 (1995), Accession No. AE000166),
- 10 • the ahpF gene of the ahpCF operon which codes for the large subunit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences USA 92(17): 7617-7621 (1995), Accession No. AE000166),

can be enhanced, in particular over-expressed.

The use of endogenous genes is in general preferred.

15 It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to the enhancement of the pgm gene, for one or more of the genes chosen from the group consisting of

- the tdh gene which codes for threonine dehydrogenase (Journal of Bacteriology 169: 4716-4721 (1987)),
- 20 • the mdh gene which codes for malate dehydrogenase (E.C. 1.1.1.37) (Archives in Microbiology 149: 36-42 (1987)),
- the gene product of the open reading frame (orf) yjfa (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- 25 • the gene product of the open reading frame (orf) ytfP (Accession Number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- the pckA gene which codes for the enzyme phosphoenolpyruvate carboxykinase (Journal of Bacteriology 172: 7151-7156 (1990)),
- 30

- the poxB gene which codes for pyruvate oxidase (Nucleic Acids Research 14(13): 5449-5460 (1986)),
- the aceA gene which codes for the enzyme isocitrate lyase (Journal of Bacteriology 170: 4528-4536 (1988)),
- 5 • the dgsA gene which codes for the DgsA regulator of the phosphotransferase system (Bioscience, Biotechnology and Biochemistry 59: 256-251 (1995)) and is also known under the name of the mlc gene,
- the fruR gene which codes for the fructose repressor  
10 (Molecular and General Genetics 226: 332-336 (1991)) and is also known under the name of the cra gene, and
- the rpoS gene which codes for the sigma<sup>38</sup> factor (WO 01/05939) and is also known under the name of the katF gene,
- 15 to be attenuated, in particular eliminated or for the expression thereof to be reduced.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are  
20 coded by the corresponding DNA, for example by using a weak promoter or a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding enzyme (protein) or gene, and optionally combining these measures.

25 By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the  
30 starting microorganism.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to the enhancement of the *pgm* gene, to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing  
5 Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention can be cultured in the batch process (batch culture), the fed  
10 batch (feed process) or the repeated fed batch process (repetitive feed process). A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction  
15 to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of  
20 the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

25 Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid,  
30 alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 25°C to 45°C, and preferably 30°C to 40°C. Culturing is continued until a maximum of L-amino acids or L-threonine has formed. This target is usually reached within 10 hours to 160 hours.

The analysis of L-amino acids can be carried out by anion exchange chromatography with subsequent ninhydrin derivation, as described by Spackman et al. (Analytical Chemistry 30: 1190-1206 (1958)), or it can take place by  
5 reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)).

The process according to the invention is used for the fermentative preparation of L-amino acids, such as, for example, L-threonine, L-isoleucine, L-valine, L-methionine,  
10 L-homoserine and L-lysine, in particular L-threonine.

The present invention is explained in more detail in the following with the aid of embodiment examples.

The minimal (M9) and complete media (LB) for Escherichia coli used are described by J.H. Miller (A Short Course in  
15 Bacterial Genetics (1992), Cold Spring Harbor Laboratory Press). The isolation of plasmid DNA from Escherichia coli and all techniques of restriction, ligation, Klenow and alkaline phosphatase treatment are carried out by the method of Sambrook et al. (Molecular Cloning - A Laboratory  
20 Manual (1989) Cold Spring Harbor Laboratory Press). Unless described otherwise, the transformation of Escherichia coli is carried out by the method of Chung et al. (Proceedings of the National Academy of Sciences of the United States of America (1989) 86: 2172-2175).

25 The incubation temperature for the preparation of strains and transformants is 37°C.

#### Example 1

Construction of the expression plasmid pTrc99Apgm

The pgm gene from E. coli K12 is amplified using the  
30 polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the pgm gene in E. coli K12 MG1655 (Accession Number

AE000172, Blattner et al. (Science 277: 1453-1462 (1997)), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany):

pgm1: 5' - CGTTGCAGACAAAGGACAAAGC - 3' (SEQ ID No. 1)

5 pgm2: 5' - GCGACCGCCCTTTTTTTATTAAATGTG - 3' (SEQ ID No. 2)

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer's instructions with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 1700 bp in size can be amplified  
10 with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is  
15 ligated with the vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Groningen, The Netherlands) in accordance with the manufacturer's instructions and transformed in the E. coli strain TOP10. Selection for plasmid-carrying cells is carried out on LB agar, to which 50 µg/ml kanamycin are added. After isolation of the  
20 plasmid DNA, the vector pCR-Blunt II-TOPO-pgm is cleaved with the restriction enzymes SpeI and XbaI and, after separation in 0.8% agarose gel with the aid of the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), the pgm fragment is isolated. The vector pTrc99A (Pharmacia  
25 Biotech, Uppsala, Sweden) is cleaved with the enzyme XbaI and ligated with the isolated pgm fragment. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 µg/ml ampicillin  
30 are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes EcoRV and PvuII. The plasmid is called pTrc99Apgm (figure 1).

Example 2

Preparation of L-threonine with the strain MG442/pTrc99Apgm

The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

The strain MG442 is transformed with the expression plasmid pTrc99Apgm described in example 1 and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar with 50 µg/ml ampicillin. The strains MG442/pTrc99Apgm and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 1.5 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{NH}_4\text{Cl}$ , 0.1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 1 g/l  $\text{KH}_2\text{PO}_4$ , 0.5 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland). 250 µl portions of this preculture are transinoculated into 10 ml of production medium (25 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 2 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.018 g/l  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 30 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. The formation of L-threonine by the starting strain MG442 is investigated in the same manner, but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction  
5 with ninhydrin detection.

The result of the experiment is shown in Table 1.

Table 1

Strain	OD (660 nm)	L-Threonine g/l
MG442	5.6	1.4
MG442/pTrc99A	3.8	1.3
MG442/pTrc99Apgm	5.4	2.4

#### Brief Description of the Figure:

- 10 • Figure 1: Map of the plasmid pTrc99Apgm containing the pgm gene.

The length data are to be understood as approx. data. The abbreviations and designations used have the following meaning:

- 15 • Amp: Ampicillin resistance gene
- lacI: Gene for the repressor protein of the trc promoter
- Ptrc: trc promoter region, IPTG-inducible
- pgm: Coding region of the pgm gene
- 20 • 5S: 5S rRNA region

• 3S: 3S rRNA region

The abbreviations for the restriction enzymes have the following meaning

- EcoRV: Restriction endonuclease from *Escherichia coli* B946
- 5 • PvuII: Restriction endonuclease from *Proteus vulgaris*
- SpeI: Restriction endonuclease from *Sphaerotilus* species ATCC 13923
- XbaI: Restriction endonuclease from *Xanthomonas campestris*

**What is claimed is:**

1. Process for the preparation of L-amino acids, in particular L-threonine, wherein the following steps are carried out:
  - 5 a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which the pgm gene or the nucleotide sequence which codes for this is enhanced, in particular over-expressed,
  - 10 b) concentration of the desired L-amino acid in the medium or in the cells of the microorganisms, and
  - c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100%) thereof
  - 15 optionally remaining in the product.
2. Process according to claim 1, wherein microorganisms in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are employed.
- 20 3. Process according to claim 1, wherein microorganisms in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.
4. Process according to claim 1, wherein the expression of
- 25 the polynucleotide which codes for the pgm gene is increased.
5. Process according to claim 1, wherein the regulatory and/or catalytic properties of the polypeptide (protein) for which the polynucleotide pgm codes are
- 30 improved or increased.

6. Process according to claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:
- 5
- 6.1 the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,
- 6.2 the pyc gene which codes for pyruvate carboxylase,
- 10
- 6.3 the pps gene which codes for phosphoenol pyruvate synthase,
- 6.4 the ppc gene which codes for phosphoenol pyruvate carboxylase,
- 15
- 6.5 the pntA and pntB genes which code for transhydrogenase,
- 6.6 the rhtB gene which imparts homoserine resistance,
- 6.7 the mgo gene which codes for malate:quinone oxidoreductase,
- 20
- 6.8 the rhtC gene which imparts threonine resistance,
- 6.9 the thrE gene which codes for the threonine export protein,
- 25
- 6.10 the gdhA gene which codes for glutamate dehydrogenase,
- 6.11 the dps gene which codes for the global regulator Dps,

- 6.12 the lrp gene which codes for the regulator of the leucine Lrp regulon,
- 6.13 the hns gene which codes for the DNA-binding protein HLP-II,
- 5 6.14 the fba gene which codes for fructose biphosphate aldolase,
- 6.15 the ptsG gene which codes for the glucose-specific IIBC component,
- 10 6.16 the ptsH gene which codes for the phosphohistidine protein hexose phosphotransferase,
- 6.17 the ptsI gene which codes for enzyme I of the phosphotransferase system,
- 15 6.18 the crr gene which codes for the glucose-specific IIA component,
- 6.19 the mopB gene which codes for chaperone GroES,
- 6.20 the ahpC gene which codes for the small sub-unit of alkyl hydroperoxide reductase,
- 20 6.21 the ahpF gene which codes for the large sub-unit of alkyl hydroperoxide reductase,

is or are enhanced, in particular over-expressed, are fermented.

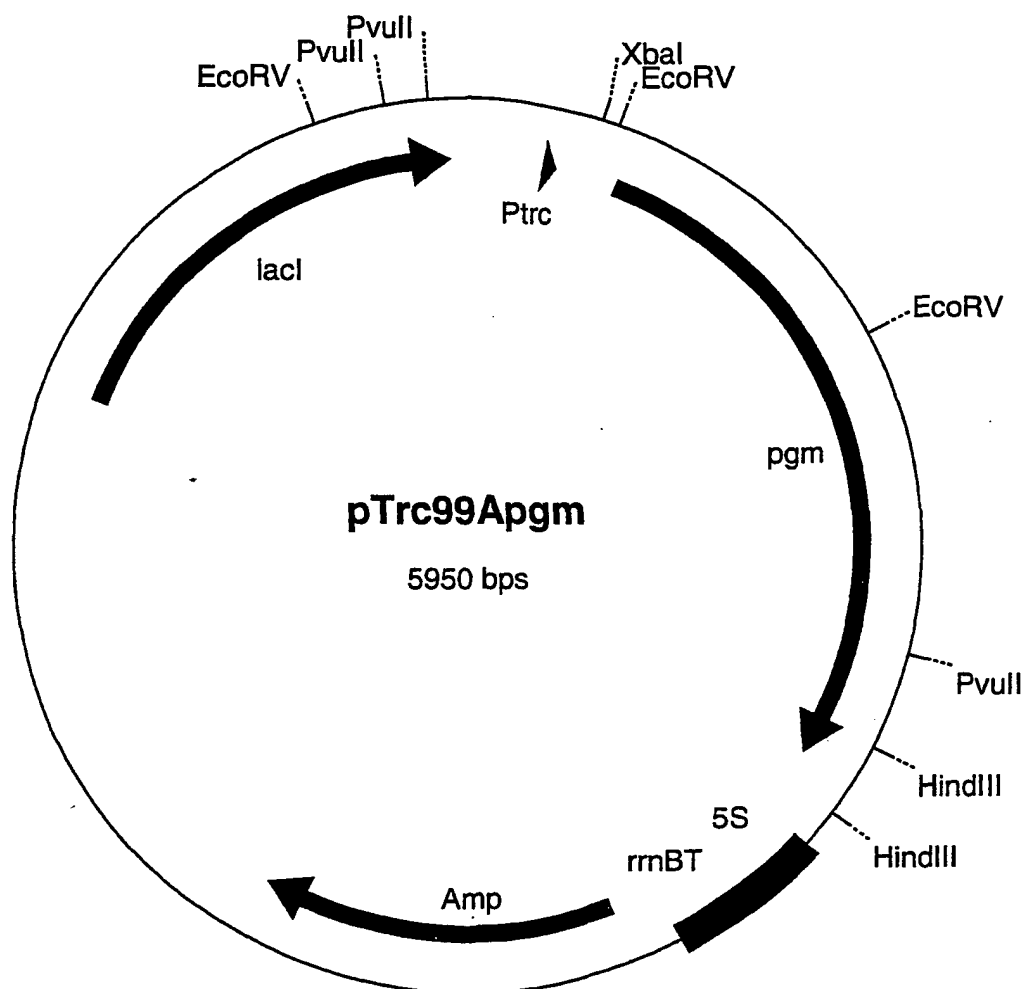
7. Process according to claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:
- 25

- 7.1 the tdh gene which codes for threonine dehydrogenase,

- 7.2 the mdh gene which codes for malate dehydrogenase,
- 7.3 the gene product of the open reading frame (orf) yjfA,
- 5 7.4 the gene product of the open reading frame (orf) ytfP,
- 7.5 the pckA gene which codes for phosphoenol pyruvate carboxykinase,
- 7.6 the poxB gene which codes for pyruvate oxidase,
- 10 7.7 the aceA gene which codes for isocitrate lyase,
- 7.8 the dgsA gene which codes for the DgsA regulator of the phosphotransferase system,
- 7.9 the fruR gene which codes for the fructose repressor,
- 15 7.10 the rpoS gene which codes for the sigma<sup>38</sup> factor

is or are attenuated, in particular eliminated or reduced in expression, are fermented.

Fig 1:



## SEQUENCE PROTOCOL

5 <110> Degussa AG  
<120> Process for the preparation of L-amino acids using strains of the Enterobacteriaceae family  
10 <130> 020095 BT  
<160> 2  
<170> PatentIn version 3.1  
15 <210> 1  
<211> 22  
<212> DNA  
<213> artificial sequence  
20 <220>  
<221> Primer  
<222> (1)..(22)  
<223> pgm1  
25 <400> 1  
cgttgcagac aaaggacaaa gc 22  
<210> 2  
<211> 27  
30 <212> DNA  
<213> artificial sequence  
<220>  
<221> Primer  
35 <222> (1)..(27)  
<223> pgm2  
<400> 2  
gcgaccgccc tttttttatt aaatgtg 27  
40

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
16 January 2003 (16.01.2003)

PCT

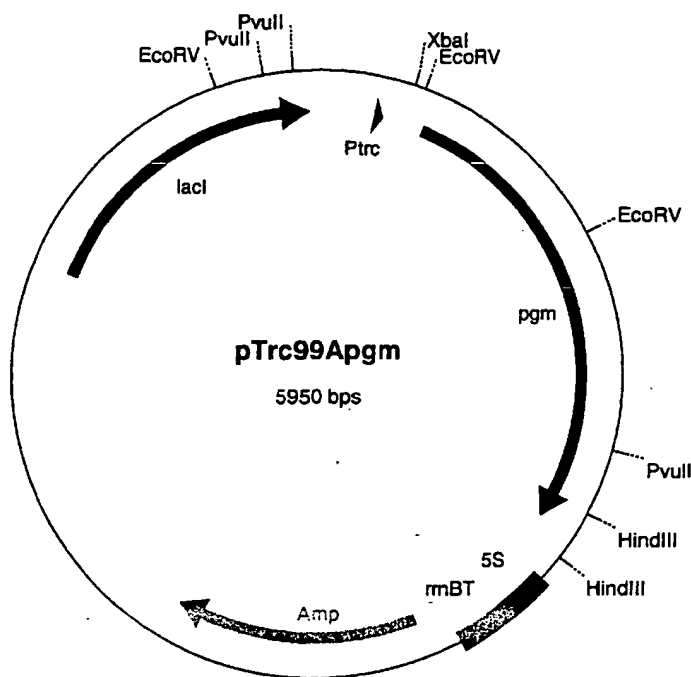
(10) International Publication Number  
**WO 03/004598 A3**

- (51) International Patent Classification: **C12P 13/06**, 13/08, 13/10, 13/12, 13/14, 13/20, 13/22, 13/24
- (21) International Application Number: PCT/EP02/06565
- (22) International Filing Date: 14 June 2002 (14.06.2002)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
101 32 946.6 6 July 2001 (06.07.2001) DE  
60/303,790 10 July 2001 (10.07.2001) US
- (71) Applicant (for all designated States except US): **DE-GUSSA AG** [DE/DE]; Bennigsenplatz 1, 40474 Düsseldorf (DE).
- (72) Inventor; and
- (75) Inventor/Applicant (for US only): **RIEPING, Mechthild** [DE/DE]; Mönkebergstrasse 1, 33619 Bielefeld (DE).
- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Declaration under Rule 4.17:**  
— of inventorship (Rule 4.17(iv)) for US only

[Continued on next page]

(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY

(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine.



WO 03/004598 A3



**Published:**

— with international search report

**(88) Date of publication of the international search report:**

18 December 2003

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 02/06565

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12P13/06 C12P13/08 C12P13/10 C12P13/12 C12P13/14  
C12P13/20 C12P13/22 C12P13/24

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, WPI Data, EPO-Internal, PAJ, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>LU MIN ET AL.: "Molecular cloning and characterization of the pgm gene encoding phosphoglucomutase of Escherichia coli." JOURNAL OF BACTERIOLOGY, vol. 176, no. 18, 1994, pages 5847-5851, XP008014250 ISSN: 0021-9193 cited in the application the whole document</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1-7



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*Z\* document member of the same patent family

Date of the actual completion of the international search

27 February 2003

Date of mailing of the international search report

26/03/2003

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.  
Fax: (+31-70) 340-3016

Authorized officer

Kools, P

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 02/06565

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SAWERS G: "THE ANAEROBIC DEGRADATION OF L-SERINE AND L-THREONINE IN ENTEROBACTERIA: NETWORKS AND PATHWAYS AND REGULATORY SIGNALS" ARCHIVES OF MICROBIOLOGY, BERLIN, DE, vol. 171, no. 1, 1998, pages 1-5, XP002953871 ISSN: 0302-8933 the whole document ---	1-7
A	CHASSAGNOLE CHRISTOPHE ET AL: "An integrated study of threonine-pathway enzyme kinetics in Escherichia coli." BIOCHEMICAL JOURNAL, vol. 356, no. 2, 1 June 2001 (2001-06-01), pages 415-423, XP002232684 ISSN: 0264-6021 the whole document ---	1-7
A	KASE H ET AL: "STUDIES ON L-THREONINE FERMENTATION PART I. PRODUCTION OF L-THREONINE BY AUXOTROPHIC MUTANTS OF VARIOUS BACTERIA" AGRICULTURAL AND BIOLOGICAL CHEMISTRY, JAPAN SOCIETY FOR BIOSCIENCE, BIOTECHNOLOGY AND AGROCHEMISTRY,, JP, vol. 35, no. 13, 1971, pages 2089-2096, XP000984541 ISSN: 0002-1369 the whole document ---	1-7
A	US 4 347 318 A (MIWA KIYOSHI ET AL) 31 August 1982 (1982-08-31) the whole document ---	1-7
A	LANDGRAF J R ET AL: "The role of H-NS in one carbon metabolism." BIOCHIMIE. FRANCE 1994, vol. 76, no. 10-11, 1994, pages 1063-1070, XP008014239 ISSN: 0300-9084 page 1064, column 1, paragraph 3 -page 1064, column 2, paragraph 1 ---	6
A	EP 0 837 134 A (AJINOMOTO KK) 22 April 1998 (1998-04-22) the whole document ---	1-7
E	WO 03 004669 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 16 January 2003 (2003-01-16) claim 6 ---	6
E	WO 03 004665 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 16 January 2003 (2003-01-16) claim 6 ---	6
-/--		

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 02/06565

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 03 004664 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 16 January 2003 (2003-01-16) claim 6 ----	6
E	WO 03 004663 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 16 January 2003 (2003-01-16) claim 6 ----	6
E	WO 03 004674 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 16 January 2003 (2003-01-16) claim 6 ----	6
E	WO 03 004675 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 16 January 2003 (2003-01-16) claim 6 ----	6
E	WO 03 004671 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 16 January 2003 (2003-01-16) claim 6 ----	6
E	WO 03 004670 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 16 January 2003 (2003-01-16) claim 6 -----	6

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 02/06565

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 4347318	A	31-08-1982	JP 1029559 B	12-06-1989
			JP 1552063 C	23-03-1990
			JP 55131397 A	13-10-1980
			DE 3012921 A1	23-10-1980
			FR 2453216 A1	31-10-1980
			GB 2049670 A , B	31-12-1980
EP 0837134	A	22-04-1998	JP 10113183 A	06-05-1998
			AU 725935 B2	26-10-2000
			AU 4096597 A	23-04-1998
			BR 9705038 A	06-04-1999
			CA 2214498 A1	15-04-1998
			CN 1182133 A	20-05-1998
			CZ 9703272 A3	17-06-1998
			EP 0837134 A2	22-04-1998
			HU 9701652 A2	28-06-1999
			PL 322611 A1	27-04-1998
			SK 139297 A3	03-06-1998
			US 5932453 A	03-08-1999
			ZA 9709229 A	11-05-1998
WO 03004669	A	16-01-2003	DE 10132946 A1	16-01-2003
			WO 03004663 A2	16-01-2003
			WO 03004669 A2	16-01-2003
			WO 03004674 A2	16-01-2003
			WO 03004670 A2	16-01-2003
			WO 03004664 A2	16-01-2003
			WO 03004598 A2	16-01-2003
			WO 03004665 A2	16-01-2003
			WO 03004671 A2	16-01-2003
			WO 03004675 A2	16-01-2003
WO 03004665	A	16-01-2003	DE 10132946 A1	16-01-2003
			WO 03004663 A2	16-01-2003
			WO 03004669 A2	16-01-2003
			WO 03004674 A2	16-01-2003
			WO 03004670 A2	16-01-2003
			WO 03004664 A2	16-01-2003
			WO 03004598 A2	16-01-2003
			WO 03004665 A2	16-01-2003
			WO 03004671 A2	16-01-2003
			WO 03004675 A2	16-01-2003
WO 03004664	A	16-01-2003	DE 10132946 A1	16-01-2003
			WO 03004663 A2	16-01-2003
			WO 03004669 A2	16-01-2003
			WO 03004674 A2	16-01-2003
			WO 03004670 A2	16-01-2003
			WO 03004664 A2	16-01-2003
			WO 03004598 A2	16-01-2003
			WO 03004665 A2	16-01-2003
			WO 03004671 A2	16-01-2003
			WO 03004675 A2	16-01-2003
WO 03004663	A	16-01-2003	DE 10132946 A1	16-01-2003
			WO 03004663 A2	16-01-2003
			WO 03004669 A2	16-01-2003
			WO 03004674 A2	16-01-2003

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 02/06565

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03004663	A	WO 03004670 A2	16-01-2003
		WO 03004664 A2	16-01-2003
		WO 03004598 A2	16-01-2003
		WO 03004665 A2	16-01-2003
		WO 03004671 A2	16-01-2003
		WO 03004675 A2	16-01-2003
WO 03004674	A 16-01-2003	DE 10132946 A1	16-01-2003
		WO 03004663 A2	16-01-2003
		WO 03004669 A2	16-01-2003
		WO 03004674 A2	16-01-2003
		WO 03004670 A2	16-01-2003
		WO 03004664 A2	16-01-2003
		WO 03004598 A2	16-01-2003
		WO 03004665 A2	16-01-2003
		WO 03004671 A2	16-01-2003
		WO 03004675 A2	16-01-2003
WO 03004675	A 16-01-2003	DE 10132946 A1	16-01-2003
		WO 03004663 A2	16-01-2003
		WO 03004669 A2	16-01-2003
		WO 03004674 A2	16-01-2003
		WO 03004670 A2	16-01-2003
		WO 03004664 A2	16-01-2003
		WO 03004598 A2	16-01-2003
		WO 03004665 A2	16-01-2003
		WO 03004671 A2	16-01-2003
		WO 03004675 A2	16-01-2003
WO 03004671	A 16-01-2003	DE 10132946 A1	16-01-2003
		WO 03004663 A2	16-01-2003
		WO 03004669 A2	16-01-2003
		WO 03004674 A2	16-01-2003
		WO 03004670 A2	16-01-2003
		WO 03004664 A2	16-01-2003
		WO 03004598 A2	16-01-2003
		WO 03004665 A2	16-01-2003
		WO 03004671 A2	16-01-2003
		WO 03004675 A2	16-01-2003
WO 03004670	A 16-01-2003	DE 10132946 A1	16-01-2003
		WO 03004663 A2	16-01-2003
		WO 03004669 A2	16-01-2003
		WO 03004674 A2	16-01-2003
		WO 03004670 A2	16-01-2003
		WO 03004664 A2	16-01-2003
		WO 03004598 A2	16-01-2003
		WO 03004665 A2	16-01-2003
		WO 03004671 A2	16-01-2003
		WO 03004675 A2	16-01-2003